*Neuropathology and Applied Neurobiology* (2001), 27, 68–76

# The mitochondrial toxin 3-nitropropionic acid induces differential expression patterns of apoptosis-related markers in rat striatum

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J. C. Vis, M. M. Verbeek, R. M. W. de Waal, H. J. ten Donkelaar and B. Kremer (2001) *Neuropathology and Applied Neurobiology* 27, 68–76

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The mitochondrial toxin 3-nitropropionic acid (3-NP) causes selective striatal lesions in rats and serves as an experimental model for the neurodegenerative disorder Huntington's disease (HD). Apoptotic cell death has been implicated for the neuronal degeneration that occurs in HD brains. The present study was designed to investigate whether the 3-NP-induced cell death in rats involves apoptosis and an altered expression of Bcl-2 family proteins. Systemic administration of 3-NP via subcutaneous Alzet pumps resulted in lesions of variable severity with neuronal loss and gliosis in the striatum. Using the terminal transferase-mediated biotinylated-UTP nick end-labelling (TUNEL) of DNA, TUNEL-positive cells exhibiting typical apoptotic morphology were detected only in the striatum of rats with a severe lesion. Furthermore, the neuronal expression of the pro-apoptotic protein Bax was strongly increased in the core of the severe lesion. Expression of the anti-apoptotic marker Bcl-2 was unchanged in this location, but was enhanced in the margins of the lesions. A moderately increased expression of both Bax and Bcl-2 was observed in dark neurones in the mild lesion and in the subtle lesion. The presence of nuclear DNA fragmentation, strong granular Bax expression and an increased Bax/Bcl-2 ratio in the centre of severe lesions suggests the occurrence of apoptotic cell death following 3-NP administration. In contrast, the dark compromised neurones observed in 3-NP-treated animals revealed an equally enhanced expression of both Bax and Bcl-2, but lacked TUNEL-labelling, and are therefore not apoptotic.

Keywords: 3-nitropropionic acid, apoptosis, dark neurones, Huntington's disease, rat, striatum

## Introduction

Apoptosis or programmed cell death is a biological phenomenon that plays a crucial role in immune regulation, normal development, and tissue homeostasis [14]. It has been suggested that an inappropriate activation of this cell death pathway is involved in chronic neurodegenerative diseases such as Huntington's disease (HD) [24]. HD is an inherited disorder and is characterized by severe atrophy of the neostriatum with marked neuronal loss and gliosis [10,34]. The precise mechanism of this selected neuronal death is unknown, but it may result from apoptosis. Interestingly, morphological evidence of apoptotic cell death has been reported in *post-mortem* HD brains [6,25,31].

Cells undergoing apoptosis show characteristic morphological features such as membrane blebbing and nuclear condensation and can be characterized biochemically by the induction of DNA fragmentation [14,28]. This DNA fragmentation can be morphologically detected using the terminal transferase-mediated biotinylated-UTP nick end-labelling (TUNEL), which specifically labels the 3′ hydroxyl terminals of DNA

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strand breaks in nuclei and apoptotic bodies [8]. However, careful assessment of the cellular morphology of TUNEL-positive cells is necessary, because the TUNEL-method could detect extensive DNA damage as the result of necrosis as well [33].

Apoptotic cell death is considered to be an active process requiring increased synthesis of particular proteins that kill the cell [27]. In recent years, many genes and proteins have been characterized that appear to regulate and execute the apoptosis pathway in the nervous system. Members of the Bcl-2 family of proteins play a major role in determining whether neurones will undergo apoptosis under conditions that promote cell death. Both Bax and Bcl-2 proteins are expressed by healthy neurones at a basal level; increased expression of Bax has been found to promote apoptosis, whereas over-expression of Bcl-2 can inhibit apoptotic cell death [16, 19,21]. The pro-apoptotic function of Bax homodimers can be directly antagonized by Bcl-2 through the formation of Bax/Bcl-2 heterodimers. Therefore, the relative expression of members of the Bcl-2 family by neurones may be indicative of apoptosis [11,12,22].

The apoptotic process takes only a few hours from start to completion. Consequently, the detection of morphological features of apoptosis may be difficult in a chronic disease like HD, in which only a few cells at any time would be expected to undergo cell death [28]. Therefore to study mechanisms of apoptotic cell death in HD, the 3-nitropropionic acid (3-NP) rat model was used [3]. In this animal model, chronic systemic administration of the mitochondrial toxin 3-NP using osmotic pumps results in selective striatal lesions, displaying characteristic neuropathological features of HD in a wide spectrum [32]. Several studies have reported that 3-NP is able to induce apoptosis in cultured neurones [4,23] and in striatal neurones of 3-NP-treated animals [1,15,26,27,30], as evaluated by TUNEL-labelling. In order to examine the possibility of apoptotic cell death, the expression of the apoptosis-related markers Bax and Bcl-2 in the striatum of 3-NP-treated rats was studied using immunohistochemistry in addition to the TUNEL-labelling.

## Materials and methods

### 3-Nitropropionic acid animal model

Five-month-old male Wistar rats of 400–450 g were used in this study. Eleven animals were treated with 3-NP (Sigma, St. Louis, MO, USA) for 4 weeks at a dose of 14 $mg.kg^{-1}.day^{-1}$ by a subcutaneous Alzet pump and were killed at the end of the treatment [3]. Three other rats became seriously ill after 9, 11 and 17 days of 3-NP administration, respectively, after which the animals were sacrificed. 3-NP was dissolved in milliQ water, adjusted to pH 7.4 and passed through a 0.2-μm filter to remove any bacterial contamination. Six control animals received normal saline.

### Histological studies

For histological examination animals were deeply anaesthesized with pentobarbital (60 mg/kg, i.p.) and perfused intracardially with 50 ml Tyrode solution, followed by 400 ml phosphate buffered 4% paraformaldehyde. Following perfusion, brains were removed and immersed in the same fixative used for perfusion at 4°C for at least 18 h. Brains were either serially sectioned on a vibratome into 50 μm free floating sections or were paraffin-embedded, and cut into 5 μm sections. Sections were stained with haematoxylin and eosin (H&E) or were stained with cresylviolet to visualize the Nissl substance.

Immunohistochemistry was performed on deparaffinized sections at intervals of 250 μm throughout the striatum using commercially available antisera. A polyclonal rabbit antibody directed against glial fibrillary acidic protein (GFAP) was obtained from Dako (Holstrup, Denmark) and was used at a dilution of 1:400 in 1% bovine serum albumin (BSA)/phosphate-buffered saline (PBS). Polyclonal rabbit antisera directed against Bax (P-19) and Bcl-2 (N-19) were both obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and were used at dilutions of 1:500 and 1:250 in 1% BSA/PBS, respectively. Prior to Bax and Bcl-2 immunohistochemistry, deparaffinized sections were subjected to a microwave treatment for 10 min in a boiling citrate solution (pH 6.0). All deparaffinized sections were incubated for 30 min in 0.2% $H_2O_2$ (Merck, Darmstadt, Germany) in PBS to quench endogenous peroxidase activity and subsequently, unspecific binding was blocked by 30 min incubation with 20% normal goat serum in PBS. Overnight incubation with the primary antibodies at 4°C was followed by incubation with a secondary biotinylated goat anti-rabbit antibody at a dilution of 1:200 in 1% BSA/PBS for 45 min. To visualize antibody binding, a standard avidin–biotin complex (Vectastain Elite, Vector Laboratories, Burlingame, CA, USA) was

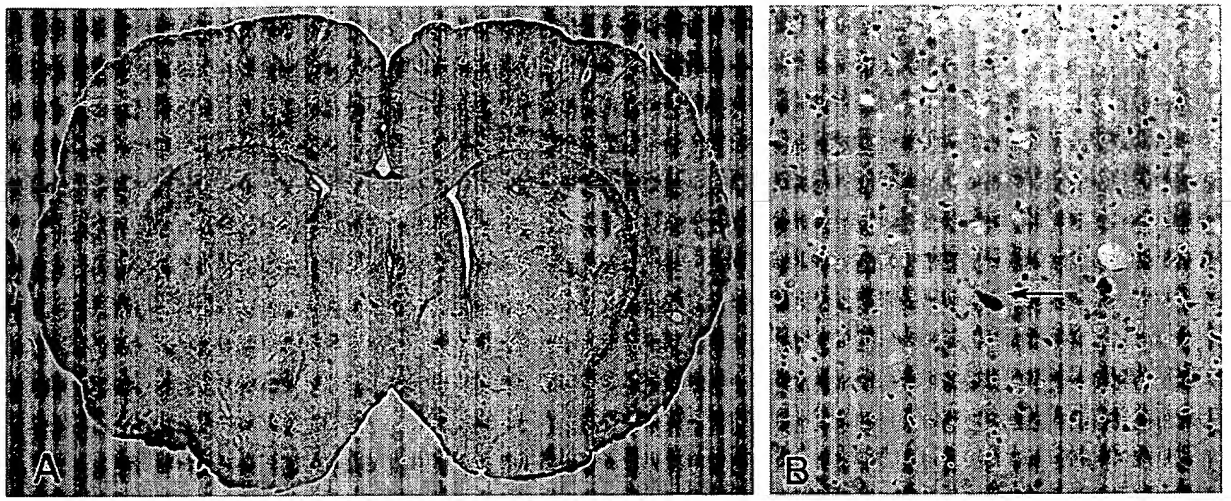


**Figure 1.** (a–b) Neuropathological characteristics of the 3-NP rat model. (a) Vibratome section of a severe lesion immunostained for GFAP, demonstrating GFAP-positive astrocytes surrounding the lesion core in which GFAP-expression is absent. Note that the neurotoxin 3-NP induced bilateral lesions in the striatum (10 ×). (b) Nissl-stained section of a severe lesion demonstrating extensive neuronal degeneration at the top right of the picture whereas compromised dark neurones (arrow) are present in the margins of the lesion at the bottom left of the picture (160 ×).

applied to the sections and 3,3′-diaminobenzidine (DAB) (Sigma) was used as a chromophore. Finally, the signal of the reaction product was enhanced by incubating sections for 5 min in 0.5% copper sulphate in saline, and sections were counterstained with haematoxylin. For the pre-absorption experiments, Bax and Bcl-2 antisera were pre-incubated with P-19 Bax blocking peptide and N-19 Bcl-2 blocking peptide (both from Santa Cruz Biotechnology), respectively, for 2 h at room temperature prior to overnight incubation of the sections.

Nuclear DNA fragmentation was visualized in deparaffinized sections using the Apoptag® kit (Intergen Company/Oncor Inc., Gaithersburg, MD, USA) containing TdT-mediated dUTP-digoxigenin 3′ nick-end labelling (TUNEL) according to the manufacturer's recommendations. TUNEL-positive cells were visualized using DAB and 0.5% copper sulphate in saline, after which the sections were counterstained using 'Kernechtrot'.

Results were obtained by microscopic evaluation of immunostained sections taken every 250 μm throughout the striatal lesion (2 mm). Immunohistochemical labelling of neurones was determined by virtue of their typical neuronal cell morphology after counterstaining with haematoxylin. Percentages of cells expressing apoptosis-related markers and of apoptotic cells were estimated by counting the number of Bax, Bcl-2 and TUNEL-positive cells in eight consecutive sections of the striatum using a 25 × objective.

## Results

### 3-Nitropropionic acid lesions in rats

Chronic systemic administration of 3-NP to rats induced bilateral striatal lesions with a marked neuronal loss and astrocytosis (Figure 1a,b). The striatal lesions have been previously described by Vis *et al.* [32] and were classified as severe ($n=3$), mild ($n=6$) or subtle ($n=5$). The severe lesion demonstrated a marked neuronal death in the lesion core and gliosis in the margins of the lesion, whereas the mild lesion was characterized by a less pronounced neuronal loss accompanied by reactive astrocytosis throughout the lesion. The subtle lesion revealed only compromised 'dark' neurones in Nissl-stained sections, which were not observed in control animals. Moreover, similar dark neurones were also detected in the mild lesion type and in the margin of the severe lesions and they were identified as medium-sized spiny neurones [32]. Additionally, in H&E and Nissl-stained sections of 3-NP-treated animals nuclear fragmentation and chromatin condensation were observed in the striatum (see below and Figure 2h). Table 1 gives an

**Table 1.** Neuropathological characteristics of lesion types found in the 3-NP rat model

| *Lesion type* | *Number of rats* | *3-NP treatment (days)* | *Neuronal loss (Nissl staining)* | *Nuclear fragmentation (H&E/Nissl)* | *Gliosis (GFAP staining)* |
|---|---|---|---|---|---|
| Subtle/DN | 5 | 28 | −, DN only | − | + blood vessels |
| Mild | 6 | 28 | +, DN | − | + + |
| Severe | 3 | 9–11–17 | Core: + + + Margin: + +, DN | Core: + Margin: − | Core: − Margin: + + |

DN, dark neurones. GFAP, glial fibrillary acidic protein. Neuronal loss: − no neuronal loss, + subtle: + + mild: + + + severe. Nuclear fragmentation: − absent; + present. Gliosis: + blood vessels: increased GFAP-expression in astrocytes surrounding blood vessels; + +: strong gliotic reaction; −: GFAP-expression is absent.

overview of the characteristic neuropathology observed in the different 3-NP lesion types.

## Bax and Bcl-2 expression in untreated and 3-NP-treated animals

Bax and Bcl-2 immunohistochemistry were performed on deparaffinized sections, taken every 250 μm throughout the striatal lesions and consistent results for each 3-NP lesion type were obtained. In all untreated animals ($n=6$), most striatal neurones revealed a diffuse perinuclear immunostaining for both Bax and Bcl-2, whereas only a few neurones expressed Bax or Bcl-2 in the cytoplasm (Figure 2a,c).

In 3-NP-treated rats with a subtle lesion ($n=5$), only scattered shrunken dark neurones were strongly immunoreactive for both Bax and Bcl-2, together with the normal perinuclear labelling for these proteins in striatal neurones. The six animals with a mild lesion demonstrated an enhanced immunostaining for both Bax and Bcl-2 in the cytoplasm of most striatal neurones compared to control animals. Interestingly, these apoptosis-related markers were strongly and predominantly expressed in the many present compromised dark neurones (Figure 2b,d).

Pronounced alterations in the expression of Bax and Bcl-2 were only observed in the striatum of the severe type of lesion ($n=3$). An increased Bcl-2 immunoreactivity was detected in several neurones at the border of the lesion, while Bcl-2 was not expressed in neurones located in the lesion core. However, oligodendrocytes, recognized by their typical morphology and present in the core of the severe lesions did express the Bcl-2 protein. The pro-apoptotic protein Bax was highly expressed in this severe striatal lesion type, and demonstrated an intense and granular immunoreactivity in approximately half of the neurones located in the lesion core (Figure 2e). Furthermore, this granular Bax-expression was different compared to the perinuclear and cytoplasmic Bax expression observed in untreated and the other 3-NP-treated animals (compare with Figure 2c,d).

Pre-absorption of the Bax and Bcl-2 antiserum with their control peptides entirely abolished the Bax and Bcl-2 immunostaining in striatal neurones of the three different lesion types. Table 2 summarizes the expression pattern of the apoptosis-related markers Bcl-2 and Bax observed in the different 3-NP lesion types.

## TUNEL-positive cells in 3-NP-treated animals

Nuclear DNA fragmentation was detected only in the lesion core of the three severely affected animals and more than a third of the cells within the striatal lesion core were TUNEL-positive (Figure 2f). TUNEL-labelling was absent in control animals and in the 3-NP-treated rats either with a subtle or mild lesion. Most TUNEL-positive cells demonstrated DNA fragmentation, typically localized in nuclei and in apoptotic bodies, which are both characteristic for apoptotic cell death (Figure 2g). Furthermore, adjacent sections stained with H&E or cresylviolet revealed apoptotic features such as nuclear fragmentation and chromatin condensation, which was only observed in the core of severe lesions (Figure 2h). The identity of the TUNEL-positive cells is not entirely clear but the marked neuronal cell loss observed in the striatum of these severely affected animals, strongly indicates that these TUNEL-positive cells are predominantly neurones. Table 2 summarizes the TUNEL-labelling and the H&E/Nissl staining observed in the different 3-NP lesion types.

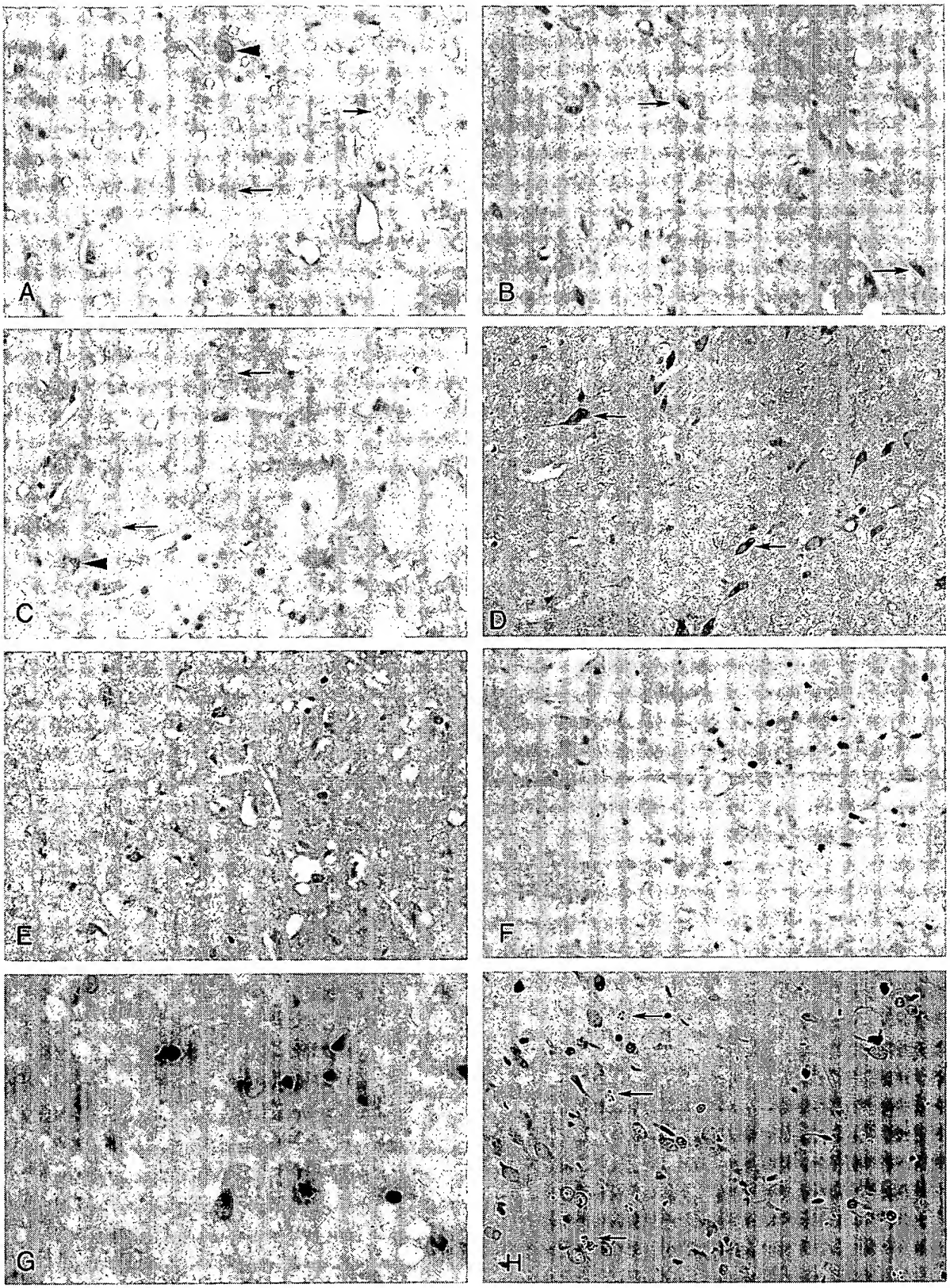

**Table 2.** Expression of apoptosis-related markers in various lesions of 3-NP rat model

| *Lesion type* | *Bcl-2* | *Bax* | *TUNEL* | *Nuclear fragmentation (H&E/Nissl staining)* |
|---|---|---|---|---|
| Subtle/DN | DN only | DN only | – | – |
| Mild | +, DN | +, DN | – | – |
| Severe | Core: * | Core: + | Core: + | Core: + |
| | Margin: + | Margin: + + | Margin: – | Margin: – |

DN, dark neurones. Bcl-2 and Bax expression in striatal neurones: *oligodendrocytes; + increased; + + intense and granular. TUNEL-positive cells: – absent; + present. Nuclear fragmentation: – absent; + present.

## Discussion

Chronic systemic administration of 3-NP to rats resulted in lesions of variable severity, classified as severe, mild, or subtle demonstrating only 'dark' neurone pathology [32]. The severe type of lesion demonstrated a marked neuronal degeneration in its centre and gliosis in the margins, whereas the mild type was characterized by a less pronounced neuronal loss accompanied by reactive astrocytosis throughout the lesion. The expression of the apoptosis-related proteins Bax and Bcl-2 was increased in all 3-NP-treated rats compared to control rats. However, the extent of the increase varied between the different lesion types. Bcl-2 was predominantly found in dark neurones that occur in all different lesion types and, in addition, was expressed in a few striatal neurones at the border of the severe lesion. The apoptosis-promoting protein Bax was also enhanced in compromised dark neurones. Besides, its expression was strongly induced in most striatal neurones of the severe lesion type. Interestingly, strong granular neuronal Bax immunoreactivity was observed in the core of the severe lesion, whereas only weak perinuclear and cytoplasmic expression was observed in untreated animals and in the other lesion types induced by 3-NP treatment. In addition, nuclear DNA fragmentation using the TUNEL-method was only observed in the lesion core of severely affected animals, whereas TUNEL-labelling was absent in the other lesions and in control rats.

It has been postulated that high levels of Bax within a cell may indicate that the cell is particularly sensitive to apoptotic cell death, owing to the formation of functional Bax homodimers that regulate and induce apoptotic cell death [13,16,19,29]. The strong granular Bax expression in the lesion core of the severe lesion type possibly reflects this enhanced Bax homodimerization. In addition to the altered expression pattern of Bax, Bcl-2 was also selectively increased in degenerating neurones and affected dark neurones. Especially, the severe lesion revealed a loss of Bcl-2 expression in the lesion core, whereas Bcl-2 was present in neurones at the border of this lesion type. A similar expression pattern for Bax and Bcl-2 was described in animal models of ischemia, revealing an increased Bax immunostaining in the centre of the lesion and elevated Bcl-2 in surviving neurones close to the penumbra [17,20,22]. According to the Bax dominant theory, Bax may have a major role in regulating apoptosis by means of Bax homodimerization, although the pro-apoptotic function of Bax can be directly antagonized by Bcl-2 through formation of Bax/Bcl-2 heterodimers [16,19]. Therefore, differential expression of these apoptosis-related proteins in neurones determines whether neurones will undergo apoptosis or not [12]. In the present study, the strong granular Bax expression and the absence of Bcl-2 in the lesion core of severe lesions may be indicative of apoptosis after 3-NP administration. Indeed, apoptotic cell death in this region was confirmed by the presence of TUNEL-labelled cells

**Figure 2.** (a–h) Expression of apoptosis-related markers in the 3-NP rat model. (a) Photomicrograph of a paraffin section from the striatum of an untreated animal showing the weak and diffuse perinuclear immunostaining for Bcl-2 in medium-sized spiny neurones (arrows). A single neurone is expressing Bcl-2 in the cytoplasm (arrowhead) (200 ×). (b) The mild type lesion demonstrates an increased expression for Bcl-2 in compromised dark neurones (arrows) (200 ×). (c) Photomicrograph of a paraffin section from a control animal showing predominantly the weak and diffuse perinuclear Bax-immunolabelling in striatal neurones (arrows), whereas only a few neurones demonstrate the cytoplasmic Bax-immunostaining (arrowhead) (200 ×). (d) Enhanced expression for Bax protein in compromised dark neurones of the mild type of lesion (arrows) (200 ×). (e) Strong and granular Bax expression in the centre of severe striatal lesions (200 ×). (f) Photomicrograph of a paraffin section demonstrating TUNEL-positive cells which were only detected in the core of the severe lesion type (200 ×). (g) TUNEL-labelled cells in the striatum of a severely lesioned animal showing DNA fragmentation typically localized in nuclei and in apoptotic bodies (400 ×). (h) Nissl-stained paraffin section from a severely lesioned animal showing a marked neuronal loss and apoptotic features such as nuclear fragmentation and chromatin condensation in the striatal lesion (arrows) (300 ×).

exhibiting typical apoptotic morphology. However, no TUNEL-labelling and no obvious change in the relative expression of Bax and Bcl-2 was detected in dark neurones and surviving striatal neurones at the border of severe lesions. This might suggest that the level of Bax protein in the cytoplasm was not yet able to induce the apoptotic process, while the upregulation of Bcl-2 expression in these neurones might reflect an active survival mechanism protecting neurones [7,11]. Still, these compromised dark neurones might undergo apoptotic cell death at a later stage or might survive and recover from this sub-lethal neuronal intoxication.

The identity of the TUNEL-positive cells observed in the severe lesions is unclear. However, these cells were located in an area of extensive neuronal degeneration, strongly suggesting that these apoptotic cells were predominantly neurones. Interestingly, the TUNEL-labelling was observed only in severely affected animals that became ill and lost 25% of their body weight. This weight loss may have led to a higher systemic dose of 3-NP and could have resulted in a faster decline of succinate dehydrogenase (SDH) activity. 3-NP irreversibly inhibits the mitochondrial SDH enzyme and the 3-NP-induced cell death is linked to mitochondrial dysfunction of the injured neurone [2,35]. Alexi *et al.* [1] reported that a rapid decline in SDH activity in vulnerable rats corresponded to a marked cell death and TUNEL-labelling in the striatum, whereas a more gradual inhibition possibly prevented acute striatal toxicity and apoptotic cell death by recruitment of compensatory mechanisms. The latter might have occurred in compromised dark neurones of the mild and subtle lesions, in which a sub-lethal 3-NP insult induced Bcl-2 expression as an active survival mechanism to protect these neurones from cell death [11]. On the contrary, in seriously ill animals that have developed a severe lesion, it was likely that the disturbed energy metabolism was highly toxic and lethal to neurones. Furthermore, since 3-NP is chemically relatively unstable at neutral pH, and will decompose to the non-neurotoxic nitrite over a few days at body temperature [9], most of the damage to the striatum may have been concentrated at the earlier part of the 4-week period of 3-NP application. Therefore, the methodology used in the present study may well have underestimated the degree of apoptotic cell death by looking too late in the process of pathogenesis. Currently, the apoptotic process is being studied at an earlier stage using intraperitoneal injections of 3-NP.

The present study demonstrates that the 3-NP-induced cell death in rats involves an apoptotic mechanism, which is associated with changes in Bax and Bcl-2 expression by neurones. It has been suggested that the specific neuronal degeneration in HD appears to result from the inappropriate activation of apoptosis [24]. Available evidence supports an apoptotic mode of neuronal death in HD, demonstrating increased levels of DNA breaks and typical apoptotic cells observed by TUNEL-labelling of HD brains. Besides the previously described neostriatal degeneration and gliosis, the occurrence of typical apoptotic cells in the striatum of 3-NP-lesioned animals demonstrates yet another similarity of this model with HD [6,25,31]. Furthermore, the 3-NP animal model demonstrated alterations in the expression pattern of the proteins Bax and Bcl-2 compared to untreated animals. Especially, the strong granular Bax expression and the absence of Bcl-2 in the lesion core of the severe lesion type suggest that the 3-NP-induced degeneration is due to apoptosis. This is consistent with several *in vivo* and *in vitro* studies reporting that 3-NP induced apoptosis in neurones as evaluated by TUNEL-labelling and mRNA expression of the Bcl-2 family of genes [1,4,15,23,26,27,30]. In addition, we observed that 3-NP-affected dark neurones demonstrated an up-regulation of both Bax and Bcl-2, which could suggest the involvement of an apoptotic mechanism. However, these dark neurones were not TUNEL-positive, implicating a neuroprotective role for the anti-apoptotic protein Bcl-2 in these 3-NP-injured neurones. The mechanism by which apoptosis is induced in HD and in animals following 3-NP administration is unknown and requires further investigation. In the 3-NP rat model the induction of apoptosis is presumably related to the effects of 3-NP on mitochondrial function, and there are several ways in which blockade of electron transport and depletion of adenosine triphosphate (ATP) might lead to apoptosis [18,27]. Both events will progressively induce a massive increase of calcium ions, which, in turn, will activate calcium-dependent cytoplasmic enzymes, including caspases that are the executioners of the apoptotic pathway [5].

In conclusion, degenerating neurones in severe lesions demonstrated distinct apoptotic features, such as TUNEL-labelling and differential expression of Bax and Bcl-2, indicating that apoptosis may be involved in the 3-NP-induced cell death. In contrast, the expression of the apoptosis-related markers Bax and Bcl-2 was increased in

affected dark neurones observed in the 3-NP model, but remained relatively equal. Besides, these cells lacked TUNEL-labelling and accordingly, the 3-NP-induced mitochondrial injury in these cells was not lethal and did not cause apoptotic cell death.

## Acknowledgements

This study was supported by a grant from the Netherlands Organization for Scientific Research (NWO-MW).

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*Received 14 August 2000*
*Accepted after revision 30 December 2000*